THE CYTOPLASMIC "PETITE" MUTATION IN SACCHAROMYCES CEREVISIAE

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Several years ago it was shown, (Bernardi *et al.*, 1968; Mehrotra and Mahler, 1969), that the mitochondrial DNAs from two genetically unrelated, acriflavine-induced, cytoplasmic "petite" mutants of *Saccharomyces cerevisiae* had a grossly altered base composition, (GC = 4%), compared to the DNAs from the parent wild-type cells, (GC = 18%). These findings unequivocally established that massive alterations in the nucleotide sequences of the mitochondrial genome may accompany the "petite" mutation, and be responsible for the altered phenotype.

Subsequent investigations, (Bernardi *et al.*, 1970), showed that the mitochondrial DNAs from three different, spontaneous, suppressive "petite" mutants" 1) had GC levels variably lower (1 .0-16.8%), than the DNAs from the parent wild-type strains; 2) had melting profiles which contained the main, low-melting component of DNAs from wild-type cells, but had lost high-melting components, again in variable degree; 3) renatured very rapidly. These results demonstrated that cytoplasmic suppressive "petite" mutants had defective mitochondrial genomes in which large segments of the parental wild-type genomes had been deleted. Four years ago, direct evidence was provided for both the deletions (Bernardi *et al.*, 1975), and the accompanying sequence amplifications (Locker *et al.*, 1974; Bernardi *et al.*, 1975).

On the basis of our early results (Bernardi *et al.*, 1970), we proposed that the deletions in the mitochondrial genomes

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of "petite" mutants arise by an excision mechanism of the Campbell type, (1962), involving site-specific, illegitimate recombination events in the AT-rich spacers of mitochondrial DNA. These spacers (Bernardi *et al.*, 1970; Bernardi and Timasheff, 1970), form 50% of the mitochondrial genome, have a GC content lower than 5%, contain short repetitive nucleotide sequences, and are likely to be endowed with some sequence homology over stretches long enough to allow recombination, (Bernardi *et al.*, 1972; Piperno *et al.*, 1972; Ehrlich *et al.*, 1972; Prunell and Bernardi, 1974). The subsequent discovery, (Bernardi, 1976a, b; Prunell and Bernardi, 1977), of other, shorter, mitochondrial DNA segments also endowed with some extent of sequence homology, the (CCGG, GGCC) clusters, raised the possibility that the site-specific, illegitimate recombination events, presumed to underlie the excision mechanism originating the defective genomes of "petite" mutants, could take place not only in the AT-rich spacer regions, but also in regions containing the other sequence elements. In either case, an interesting aspect of the model outlined above is that it attributes the instability of the mitochondrial genome of yeast, (the spontaneous "petite" mutation has a rate of 1-5% per generation), to the existence in each genome unit of a number of nucleotide sequences endowed with enough homology as to allow site-specific, illegitimate recombination events to take place.

In the present work, we have intestigated the molecular mechanisms underlying the "petite" mutation by studying in some detail the organization of the mitochondrial genome of several "petite" mutants.

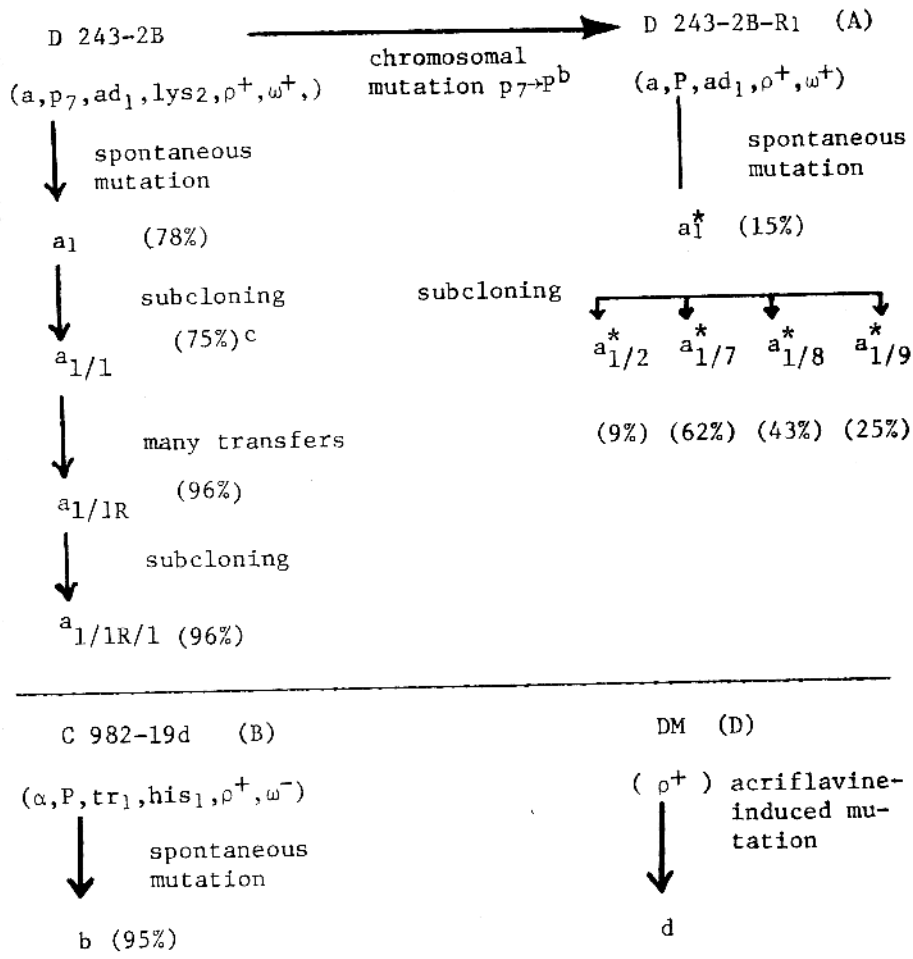
Preliminary reports of this work have been presented during the past several years (Bernardi *et al.*, 1975; 1976; Bernardi, 1975, 1976a, b).

ANALYSIS OF MITOCHONDRIAL DNA STRUCTURE

The genetic relationships among the "petite" yeast strains used are summarized in Table I. The reader is referred to previous publications from our laboratory for additional information on these "petites" and their parental wild-type strains, and for the methods employed. It is worth noting that all the "petite" mutants investigated were spontaneous, except for strain d, which was an acriflavine-induced "petite".

The experimental work to be reported here essentially consisted in studying the degradation with restriction enzymes of three "petite" genomes of low complexity and their hybrid-

TABLE 1. GENETIC RELATIONSHIPS AMONG THE YEAST STRAINS USED[a]



[a]Values in parentheses indicate the suppressibility.

[b]The $p_7 \rightarrow P$ revertant may not be a back mutation *sensu stricto* but a suppressor mutation (Mounolou, 1967).

[c]The suppressibility of this strain was determined by Faurès-Renot *et al.* (1974).

ization to restriction fragments from the corresponding parental genomes; in addition, we studied the restriction patterns of one heterogeneous population of "petite" genomes and of some of its sub-clones. A complete account of this work, which

involved the use of other techniques and of other "petite" strains, will be reported elsewhere.

Strain d. The mitochondrial DNA from this acriflavine-induced "petite" had a GC content of only 4% (Bernardi *et al.*, 1968). No degradation could be obtained with any of 9 restriction enzymes tested: EcoRI, HindII + III, HpaII, HaeIII, HhaI, TaqI, MboI, HinfI, AluI; (HpaII and HaeIII will be indicated as Hpa and Hae hencefrom). The DNA from strain d hybridized to 1 Hpa band of 1.14×10^6 and 1 Hae band of 1.15×10^6 of the DNA from the parent strain D (fig. 1).

Strain b. Only 2 of the 9 restriction enzymes tested, Hpa and AluI, were able to split this mitochondiral DNA (GC= 13.0%; Bernardi *et al.*, 1970). Each enzyme released a single fragment, having a molecular weight of 5.6×10^5 (fig. 2). Partial digests were characterized by a series of fragments having molecular weights which were exact multiples, (within 1%, or 8 base pairs), of the basic unit of 5.6×10^5 (see Bernardi, 1975; Bernardi *et al.*, 1975). Digestion with both Hpa + AluI produced a fragment 3×10^4 smaller in molecular weight than the basic unit of 5.6×10^5. These results (fig. 2) clearly show that the genome of "petite" strain b is made up of a tandem repetition of the 5.6×10^5 basic unit; the very simple restriction map of such unit is shown in fig. 3. The DNA from "petite" strain b hybridized mainly to 1 Hpa band, MW = 5.6×10^5, and 1 Hae band, MW= $7.6.10^5$, of the DNA from the parental strain B. Weaker hybridization to 2 (or 3) other Hpa and 3 other Hae bands was also observed, as well as very weak hybridization to other bands (fig. 1).

Strain $a_{1/1R/1}$. Only 3 out of 19 restriction enzymes tested, (those mentioned above plus BglI, BglII, SmaI, BamHI, HpaI, KpnI, PstI, SalI, XbaI, XhoI), were able to degrade this mitochondrial DNA. It was converted by MboI into a single fragment of 5.80×10^5, by Hae into 2 fragments of 4.9×10^5 and 0.98×10^5, and by Hpa into 3 fragments of 3.0×10^5, $1.80 \times\times 10^5$ and $1.06 \times\times 10^5$ (fig. 2). A study of partial and double digests led to the restriction map of the basic unit shown in fig. 3. Hybridization of the DNA from "petite" $a_{1/1R/1}$ to Hae and Hpa digests of the DNA from the parental wild-type strain was mainly with fragments having the same molecular weight as the Hae and Hpa fragments from the "petite" DNA (fig. 1). In addition 5-6 bands in both Hae and Hpa digests also showed significant hybridization.

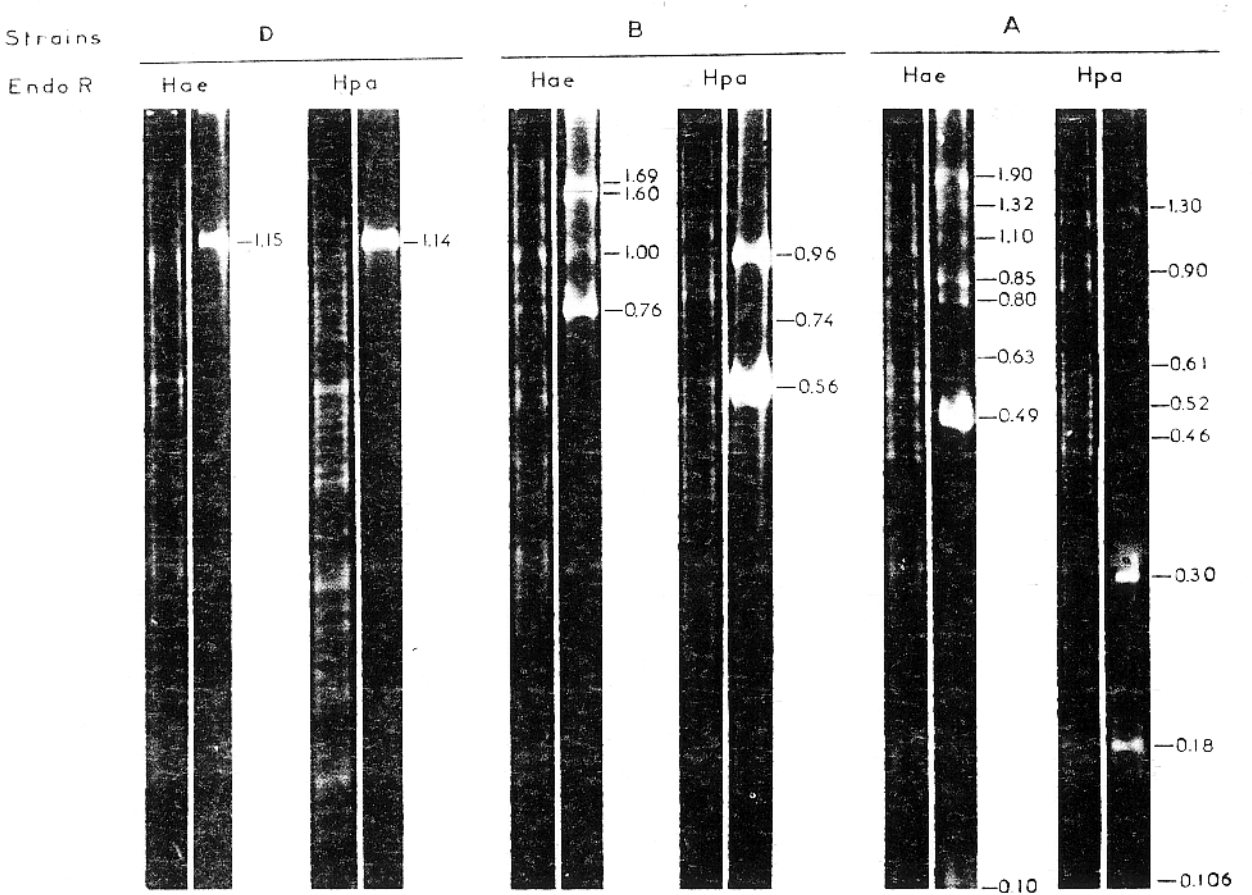


FIGURE 1. Hybridization patterns of mitochondrial DNAs from strains d, b and a to HpaII and HaeIII restriction fragments of mitochondrial DNAs from parental wild-type strains D, B and A. Molecular weights of the bands (in millions) are indicated.

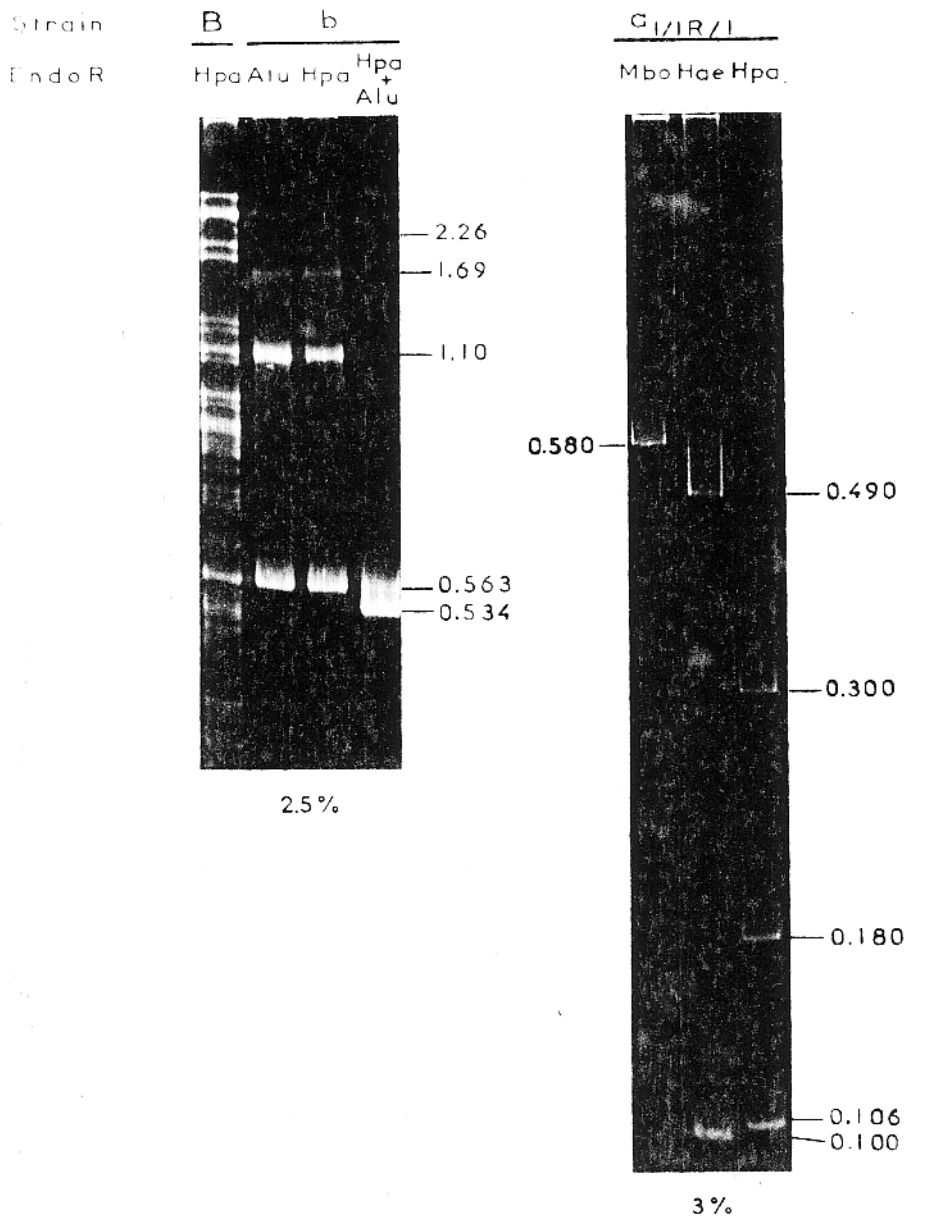


FIGURE 2. Restriction patterns of mitochondrial DNAs from "petite" strains b and $a_{1/1R/1}$. Restriction endonucleases and polyacrylamide concentrations are indicated at the top and bottom, respectively, as well as the molecular weights (in millions) of the bands.

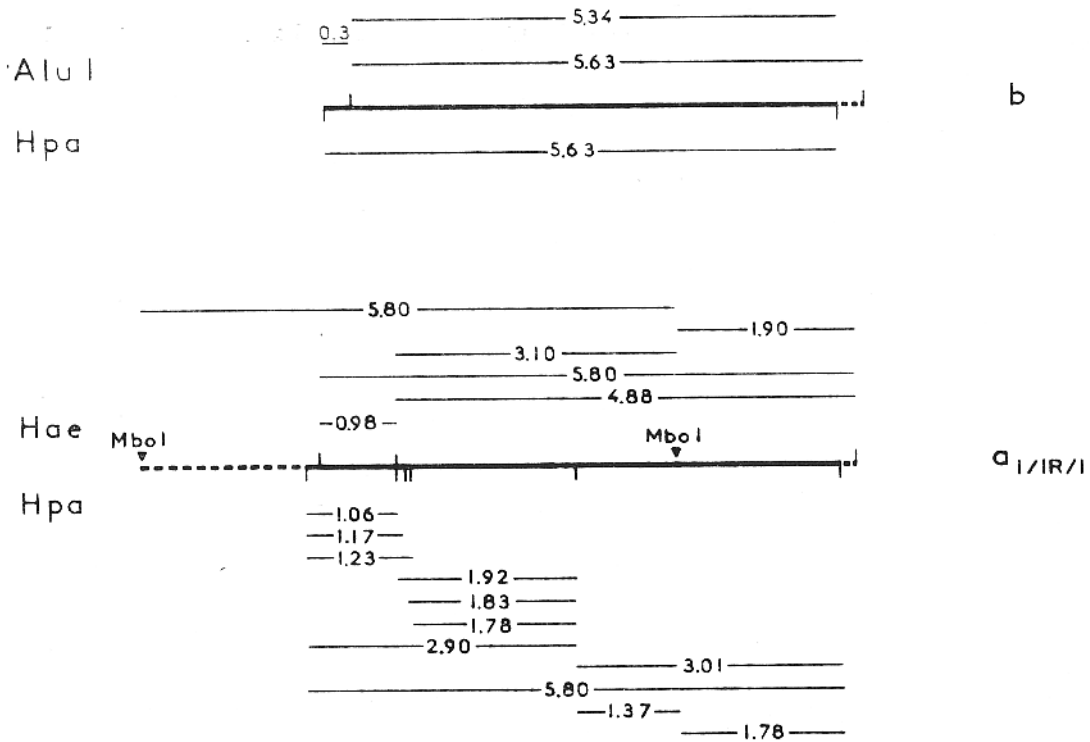


FIGURE 3. Restriction enzyme maps of the repeating units of the mitochondrial genomes of "petite" strains b and $a_{1/1R/1}$.

Strain a_1^* and its sub-clones. The Hae and Hpa patterns of the mitochondrial DNA from strain a_1^* (fig. 4) was characterized by the following features: 1) roughly one third of the bands from strain A were missing; this corresponds to a deletion of about 27 million daltons of wild-type DNA. 2) almost all bands possessed mobilities identical to those of the DNA from the parent strain A; in fact, only 1 Hpa band out of 53, and 1 Hae band out of 42, possessed different mobilities, 3) some bands showed higher intensities and some weaker intensities than the corresponding bands from strain A.

The restriction patterns of four subclones of strain a_1^*, (fig. 4), as well as differences in their suppressibility (Table I), confirm the heterogeneity of the mitochondrial genome in strain a_1^* indicated by its restriction pattern. All or nearly all of the bands present in a given sub-clone represented a sub-set of the bands of a_1^*; very few bands not present in A nor a_1^*, were also present.

THE ORGANIZATION OF THE "PETITE" GENOME

The results reported indicate that four different situations exist in the nucleotide sequences at the ends of the repeating units in the "petite" genomes investigated here.

i. The mitochondrial genome of "petite" $a_{1/1R/1}$ is formed by the tandem repetition of a DNA segment which is delimited by two Hae-Hpa clusters; these are the clusters of GGCC and CCGG detected by Prunell and Bernardi (1977); the repeat unit also contains one additional Hae-Hpa cluster, one isolated Hpa site and one MboI site. If the suggestion that GC-rich clusters are contiguous to Hae-Hpa site clusters (Prunell and Bernardi, 1977) is correct, then the repeat unit of the mitochondiral genome of "petite" $a_{1/1R/1}$ should also contain 1 to 3 GC-rich clusters.

The main hybridization of this "petite" DNA is to 2 Hae fragments and 3 Hpa fragments of the DNA from the parental wild-type cells; these fragments have exactly the same size as those obtained from the "petite" DNA. The Hae and Hpa fragments of the latter account for the totality of the "petite" genome (except for joining oligonucleotides) as shown by the absence of other bands on the gels under conditions where the smallest double-stranded fragment (stoichiometric with the main fragments) could be detected, and also by the partial hydrolysates, which can be accounted for in terms of known fragments.

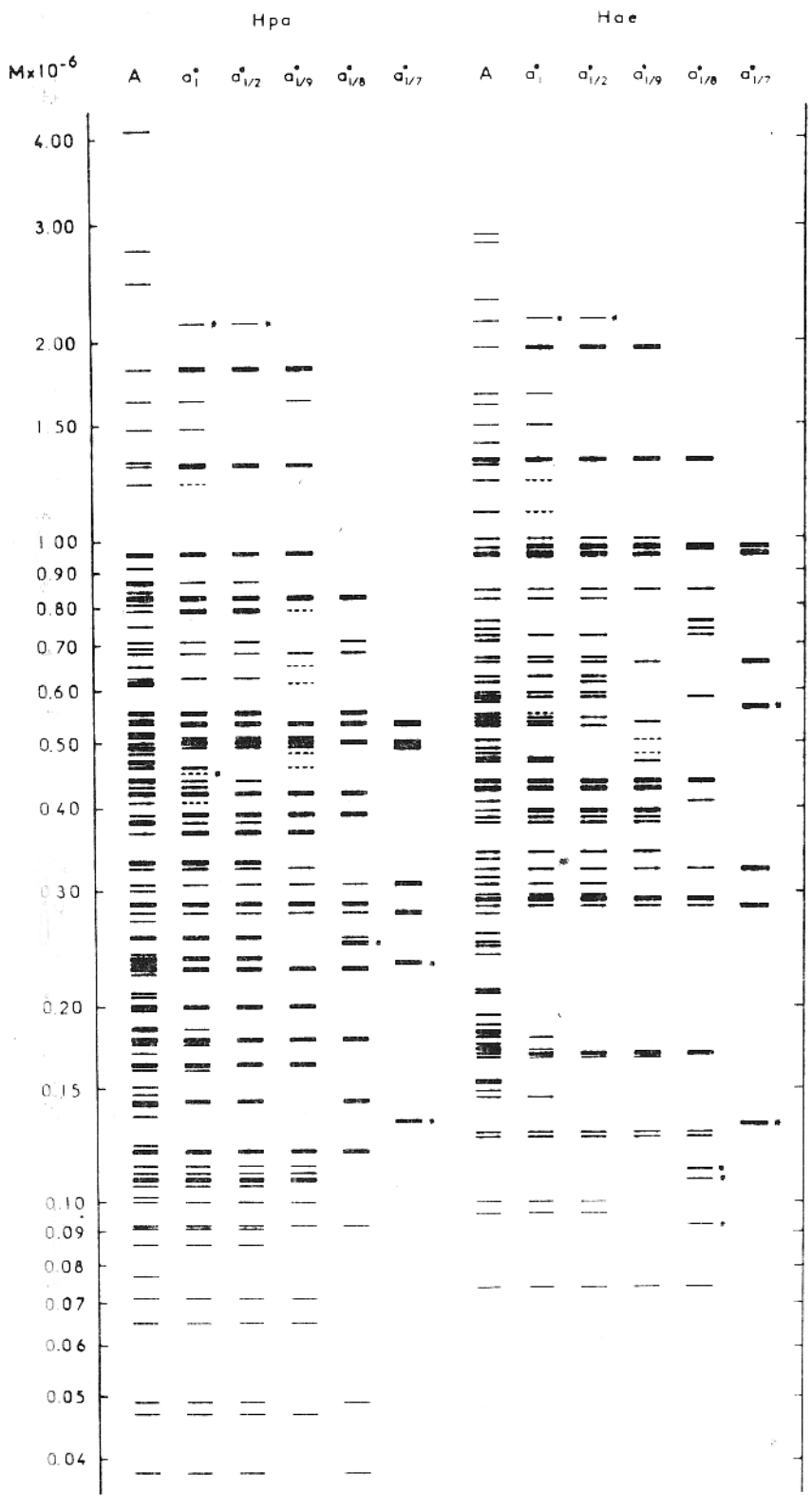

Hpa
Hae
M x 10⁻⁶
A
a₁*
a₁/₂*
a₁/₉*
a₁/₈*
a₁/₇*
4.00
3.00
2.00
1.50
1.00
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.15
0.10
0.09
0.08
0.07
0.06
0.05
0.04

FIGURE 4. Scheme of HaeIII and HpaII band patterns of mitochondrial DNAs from strains A, a_1^+ and its sub-clones. Line thicknesses indicate band intensities; broken lines indicate faint bands. Asterisks indicate bands not present in the DNA from the parental wild-type strain.

These findings strongly indicate that excision of the repeating unit of $a_{1/1R/1}$ from the parental wild-type genome did take place at two Hae-Hpa site clusters (case(a) in fig. 5). The most likely primary mechanism in this case can be considered to be a site-specific, illegitimate recombination at two Hae-Hpa site clusters located on the same or on two different genome units. In the first case, the nucleotide sequences involved in the process should correspond basically to inverted repeats; in the second case, to direct repeats.

The Hae-Hpa site clusters, (Prunell and Bernardi, 1977), are present in the wild-type genomes in a large number (about 60), are scattered all over the genome, and contain a number of symmetrical sequences, (the Hae and Hpa sites GGCC and CCGG), which might themselves be arranged symmetrically in the clusters. We suggested, (Prunell and Bernardi, 1977), that these sequences, as well as the contiguous GC-rich clusters, might have sufficient sequence homology to permit site-specific illegitimate recombination. The spurious hybridization of the DNA from $a_{1/1R/1}$ with fragments of wild-type DNA which do not correspond to the excised segment is indicative, in our opinion, of the existence of such partial homology.

As a result of illegitimate recombination, one would expect to see slight changes in the length of the clusters in the "petite" genome, compared to the wild-type genome. Since the clusters are short, (on the average 35 base pairs for the Hae-Hpa site clusters and 70 base pairs for the GC-rich clusters; Prunell and Bernardi, 1977), and are partly destroyed by Hae and Hpa digestion, these changes would not be very evident.

If one recalls that almost all Hae sites in the DNAs from wild-type cells are present in Hae-Hpa clusters (Prunell and Bernardi, 1977), then the presence of only a few "new" Hae fragments having different sizes compared to the corresponding parental ones in the DNAs from heterogeneous populations of spontaneous "petites" (fig. 4) very strongly suggests that the situation found for a $a_{1/1R/1}$ occurs frequently.

ii. The case of the genome from "petite" mutant b differs from that discussed above in that the repeat unit has the same

length as a Hpa fragment of the parental, wild-type genome, but is considerably shorter (by 2×10^5), than the corresponding Hae fragment. This means that only one and not both ends of the repeat may correspond to Hae-Hpa site clusters. Thus, if the original event was, as for a $_{1/1R/1}$, an unequal crossing-over at two Hae-Hpa site clusters, one must assume that a secondary event has eliminated part of the excised segment. A more detailed knowledge of the organization of the mitochondrial genome of b is required to better understand the excision event in this case (Fig. 5 (b)).

iii. Another situation is represented by "petite" genomes which are either formed by tandem repeats of "new" bands or, possibly, by tandem repeats of "old" and "new" bands. In this case, either one or both sites involves in the primary recombination event would be different from Hae or Hpa sites, according to scheme (c) of fig. 5. In this case we favor the idea that the site(s) different from the Hae-Hpa cluster is (are) located on spacer sequences. This case is different from the previous one since neither of the ends of the repeat unit of b DNA originates from a spacer region (Prunell, 1976).

iv. The last case is that of "petite" mutant d. In this case, the hypothetical repeat unit carries either the totality or a fraction of the seryl-tRNA gene (Carnevali *et al.*, 1973); no Hae or Hpa sites are present and over 90% of the hypothetical repeat unit is made up of spacer sequences, as judged from results obtained with micrococcal nuclease digests. It is evident that in this case, if the original event was one of those postulated above, considerable sequence rearrangements have occurred; alternatively, the original event was different from those described. In any case, we consider it significant that this "petite", in contrast to the others considered above, is the result of mutagenization with acriflavine. It is well known that the great increase in "petite" formation upon mutagenization, (from a few percent to 100% per generation), is accompanied by a fragmentation of the wild-type DNA, (Goldring *et al.*, 1970). Investigations carried out in other laboratories concerning "petite" mutants induced by ethidium bromide confirm the idea that such "petites" fall into more complex excision patterns compared to those found here for the genome of $a_{1/1R/1}$ (Heyting and Sanders, 1976).

SOME GENERAL CONSIDERATIONS

If we consider the case of $a_{1/1R/1}$ to be typical, and we assume that its excision has occurred by an internal crossing-over process involving site-specific, illegitimate recombina-

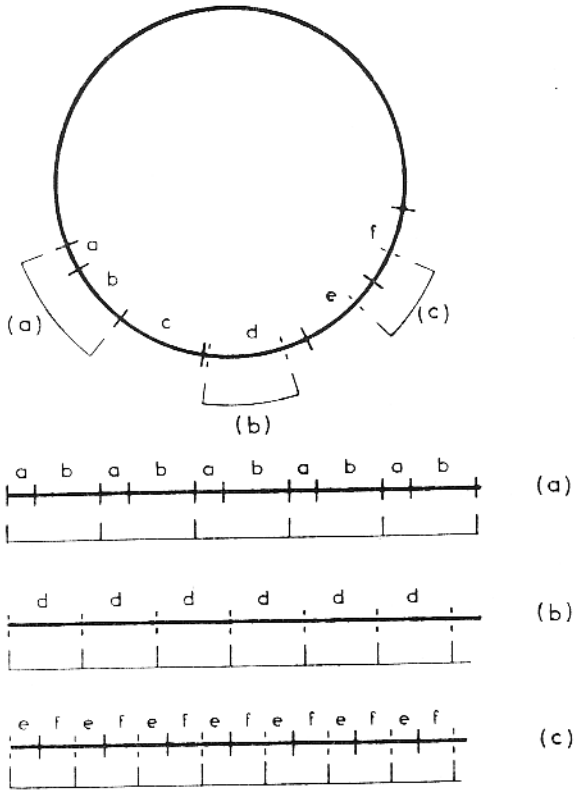


FIGURE 5. Scheme for the excision of the repeat unit of the mitochondrial genomes of "petite" mutants by a site specific, illegitimate recombination mechanism. This may be viewed as an intra- or an interchromosomal process. The circle represents the wild-type mitochondrial genome, full bars Hae-Hpa site clusters, broken bars other sites (see Text).

tion at two Hae-Hpa site clusters, it is clear that the primary event in the sponteneous "petite" mutation is very similar to the excision of lambda prophage from the *E. coli* chromosome or to the dissociation of a transposon from its host plasmid. In this case, the Hae-Hpa site clusters play the same role as the insertion sequences delimiting the bacterial transposon. Apparently, this role can also be played by other sequences endowed with similar properties, conceivably by sequences in the AT-rich spacers and in the GC-rich clusters, but never by the unique sequences of mitochondrial genes. In any case, the primary event of the "petite" mutation: 1) is accompanied, or followed, by the tandem amplification of the excised segment; 2) yields a genome unit capable of replication; 3) may

conceivably take place not only through an internal recombination process, (namely, a recombination within a single genome unit), but also through an external recombination process involving two different genome units, and 4) is the result of an excision process promoted by the presence of nucleotide stretches which share sequence homology and are present in a number of copies per genome unit, as originally postulated.

Current work in our laboratory aims at shedding light on two very important problems raised by the present work: 1) the primary structure of the sequences involved in the primary excision mechanism leading to the "petite" mutant, as well as in other recombinational events occurring in the divergence of wild-type mitochondrial genomes (Prunell *et al.*, 1977) and in crosses of wild-type cells (Fonty *et al.*, 1978), and 2) the recombination of "petite" genomes with either wild-type genomes or other "petite" genomes; this process may be seen as the equivalent of the translocation of a transposon from one plasmid to another one or to the insertion of lambda DNA into the *E. coli* genome.

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